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14. ABSTRACT:

The ultimate goal of this program is to discover selective and potent drugs targeted to prevent or relieve the neurotoxic actions of botulinum neurotoxin (BoNT) A. A major goal of this program is the identification of open channel blockers as a single class of drugs that would be effective against all BoNT isoforms. We consider the BoNT channel as a validated target for intervention aimed to inhibit the translocation of the light chain into the cytosol and therefore to attenuate the BoNT neurotoxicity. The major focus thus far has been the implementation of a reliable and robust high-throughput screen for blockers specific for BoNT using Neuro 2A cells in which BoNTA forms channels with similar properties to those previously characterized in lipid bilayers. The immediate task during the present reporting period involved the detailed characterization of the channel and chaperone activity of BoNTA on Neuro2A cells.

15. SUBJECT TERMS
Botulinum neurotoxin; drug discovery; ion channels; channel blockers; combinatorial chemistry

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Table of Contents

INTRODUCTION

This program examines innovative approaches and powerful new technologies to identify selective and potent agents directed to prevent or relieve the neuroparalytic toxic actions of botulinum toxin A (BoNTA)[1]. The focus is on the protein-conducting channel of BoNTs as a validated target to screen for inhibitors of the translocation of the light chain into the cytosol and therefore to attenuate the BoNT neurotoxicity. The key of the program is based on our discovery that the heavy chain (HC) of BoNT acts as both a protein-conducting channel and a transmembrane chaperone for the light chain (LC) to ensure a translocation competent conformation during its transit from the acidic endosome into the cytosol - its site of action[2]. This is an exciting time to focus on innovative technologies to uncover lead compounds that may represent a potential new generation of useful and safe antidotes for BoNTs.

Here, we focus on the progress concerning the implementation of a neuronal system amenable to characterize the protein-conducting channel and chaperone activities of BoNT under conditions which closely emulate those prevalent at the endosome, and which are relevant to the neurotropism and neuroparalytic effects of BoNTs.

BODY

The protein-conducting channel and chaperone activities of *Clostridial botulinum* neurotoxin (BoNT) A were investigated in Neuro 2A neuroblastoma cells under conditions that closely emulate those prevalent at the endosome. The results of this program were recently published in *Neurotoxicity Research,* Volume 9, pp. 93-100, 2006. Attached please find this publication in pdf format.

Work in progress is directed to assess the role of the heavy chain “belt” in the translocation process. Constructs were designed to produce a “beltless” heavy chain; the protein will be produced and the protein-conducting channel activity will be characterized under identical conditions as those used for the wild type holotoxin and heavy chain.

A salient feature of the BoNT channel is that it is closed at positive voltages under conditions in which the orientation and the magnitude of the pH gradient, as well as the polarity and magnitude of the membrane potential compare fairly well with those prevailing across the endosomal membrane: pH 5.3 and positive potential on the compartment containing the BoNT and pH 7.0 and negative potential on the opposite compartment. This suggests that the BoNT heavy chain channel would be closed in the endosome until it is gated by the BoNT light chain to initiate its translocation across the membrane into the cytosol. The neuroblastoma cell line appears, therefore, to be a suitable system to characterize the BoNT channel and to pursue evaluation of plausible strategies for targeted drug delivery thereby minimizing the requirement for *in vivo* animal testing.

CONCLUSIONS

The finding that the heavy chain operates as a transmembrane protein-conducting channel which is occluded by the light chain during transit, and open after completion of translocation and release of cargo, has outlined a novel way of thinking about BoNT neurotoxicity, shifting the focus of attention on its translocation within cells rather than on the protease activity of the light chain, which is known not to be toxic unless it is internalized.

This new paradigm has received a lot of visibility (Nature Structural Biology News and Views, The Lancet, The Scientist, BioWorld Today). By invitation, the results have been presented at the 2005 Fifth International Conference on the Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins, held in Denver, CO June 22-25, and at the 2005 IBRCC, International Botulism Research Consortium, in Baltimore, MD. December 5-8.

BoNT is one of the most feared biological weapons of the 21st century: One gram of aerosolized or food-borne BoNT would kill more than 1 million people. A major outcome of our program would be a blueprint to uncover blockers of the protein-conducting channel of BoNT which may be developed into prophylactic agents.

KEY RESEARCH ACCOMPLISHMENTS

- A key step in the intoxication by BoNT is the translocation of internalized toxin across intracellular membranes to reach its cytosolic targets. A fundamental discovery was the demonstration that the heavy chain acts as both a protein-conducting channel and a transmembrane chaperone for the light chain protease to ensure a translocation competent conformation during transit from acidic endosomes into the cytosol. Thus, the stage is set for pursuing the identification of channel blockers specific for the BoNT heavy chain.
- A high-throughput screen for the search of compounds that modulate or block the channel activity of BoNT on Neuro 2A cells is under evaluation.

REPORTABLE OUTCOMES

- Publications

Appendix # 1: Fischer, A. and Montal, M. Characterization of *Clostridial botulinum* neurotoxin channels in neuroblastoma cells. *Neurotoxicity Research,* **9**:93-100, 2006.

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2. Koriazova, L. K. & Montal, M. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat Struct Biol* **10**, 13-8 (2003).

APPENDICES

Appendix #1

Fischer, A. and Montal, M. Characterization of *Clostridial botulinum* neurotoxin channels in neuroblastoma cells. *Neurotoxicity Research,* **9**:93-100, 2006.

DAMD17 02-C-01-6. Amendment to Final Report dated September 2006

As requested, I am enclosing the amendment to the final report of the above-referenced grant. The request entails a description of work performed to identify small-molecule channel blockers of botulinum neurotoxin (BoNT) serotype A.

Our objective was to screen small-molecules known or suspected to act as channel blockers on the BoNT heavy chain (HC) channel reconstituted in lipid bilayers. Open-channel blockers are small molecules that enter the channel and occlude the passageway through interaction with the protein main chain or side chains exposed to the channel lumen. We discovered that numerous families of molecules known to block cation-selective channels also block the BoNT HC channel which include drugs currently used in humans (Fig. 1)[1]: amantadine, an anti-influenza drug that blocks the channels formed by the influenza virus M2 protein[2] and its derivative, memantine, which blocks the NMDA receptor channel[3], and is approved by the FDA for the treatment of dementia. The antipsychotic chlorpromazine[4], an open channel blocker of the nicotinic acetylcholine receptor (nAChR) channel[5], and numerous local anesthetics related to lidocaine, known to block Na^+ channels and nAChR channels[1]. The anti-malarial drugs chloroquine and quinacrine affect the intracellular processing of BoNTs by collapsing the pH gradient across endosomes[6,7]. Chlorpromazine, quinacrine and memantine, in addition, cross the blood-brain barrier.

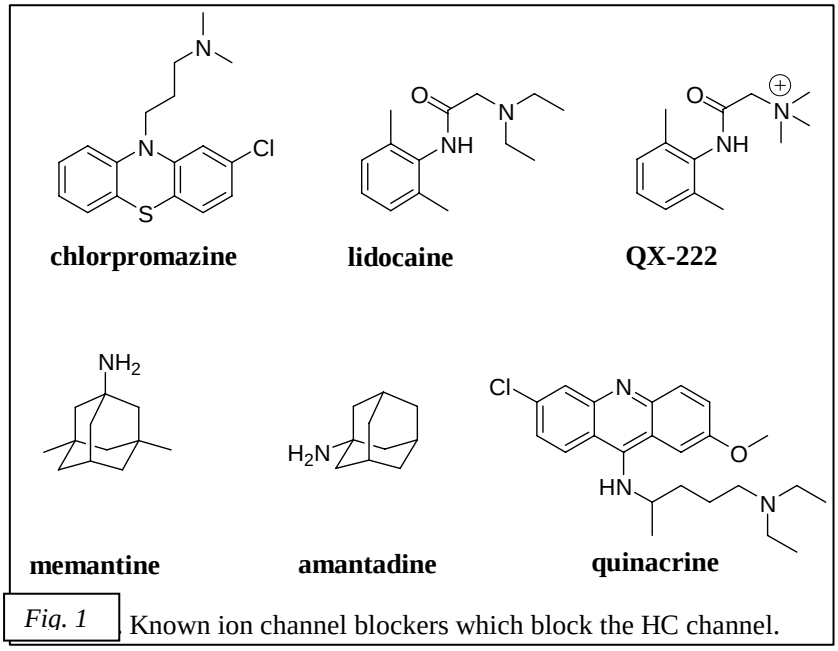

Fig. 1 Known ion channel blockers which block the HC channel.

We screened numerous open channel blockers against the BoNT reconstituted in lipid bilayers using the single channel recording assay[8]. A representative record obtained at -100 mV for QX-222 (structure displayed in Fig. 1) is shown in Fig. 2. In the absence of drug (panel A), the occurrence of up to five BoNT channels with a $\gamma \sim 110$ pS are clearly discerned; downward current deflections indicate channel opening. The current histogram shown on the right is well fitted with five Gaussian curves corresponding to five independent BoNT channels in the membrane undergoing transitions from closed to open states. In contrast, in the presence of 40 μM QX-222 (panel B), the pattern of channel activity is drastically altered: the frequency of openings is reduced and long quiescent periods dominate the records. The corresponding histogram displays a single

distribution corresponding to the closed state, a feature of channel block. This analysis provides proof-of-principle for the screen of small-molecules blockers.

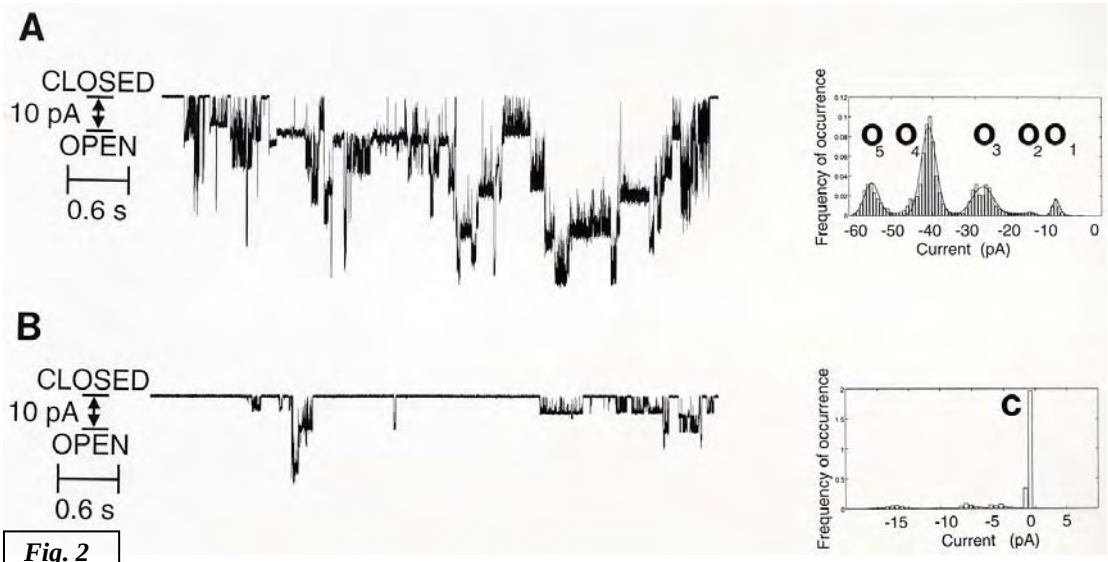

Fig. 2

Concluding Remarks. This project was conceived on the realization that development or discovery of blockers of the protein-conducting channel of BoNT, a key element in the process of neurotoxicity, is of paramount significance. The results validate the endeavor to identify structural blueprints for the design of a realistic BoNT antidote.

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Neurotoxicity Research, 2006, VOL. 9(2,3). pp. 93-100

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Characterization of *Clostridial botulinum* Neurotoxin Channels in Neuroblastoma Cells

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The channel and chaperone activities of *Clostridial botulinum* neurotoxin (BoNT) A were investigated in Neuro 2a neuroblastoma cells under conditions that closely emulate those prevalent at the endosome. Channel activity occurs in bursts interspersed between periods of little or no activity. The channels are voltage dependent, opening only at negative voltages. Within bursts, the channel resides preferentially in the open state. The channels open to a main conductance of 105 ± 5 pS or 65 ± 4 pS in 200 mM CsCl or NaCl, respectively. The BoNT channels display a conspicuous subconductance of 10 ± 2 pS. The neuroblastoma cell line appears, therefore, to be a suitable system to characterize the BoNT channel and to pursue evaluation of plausible strategies for targeted drug delivery thereby minimizing the requirement for *in vivo* animal testing.

Keywords: Botulinum neurotoxin; Channels; Chaperones; Targeted drug screen; Protein translocation

INTRODUCTION

Chemoprophylaxis and therapeutic intervention of the deadly intoxication by botulinum neurotoxins requires understanding the mechanisms by which it abrogates neurotransmitter release. Biochemically, BoNTs have two disulfide linked chains: a light chain of ~50 kD and a heavy chain of ~100 kD; structurally, BoNT consists of three domains (Lacy *et al.*, 1998; Lacy and Stevens, 1999; Schiavo *et al.*, 2000; Swaminathan and Eswaramoorthy, 2000): The N-terminal light chain is the catalytic domain whereas the heavy chain encompasses the translocation domain (the N-terminal half) and the receptor-binding domain (the C-terminal half). Our aim is to elucidate the fundamental molecular mechanisms that confer to BoNT its exquisite ability to move efficiently and selectively within neurons exploiting such tri-modular organization. An immediate objective is to understand the intricacies of intracellular trafficking of BoNT that may disclose novel pathways to escort, target, and insert it into membranes. This information may prove diagnostic in identifying unsuspected sites for intervention and plausible strategies for targeted drug delivery.

BoNTs enter sensitive cells *via* receptor-mediated endocytosis (Hoch *et al.*, 1985, Schiavo *et al.*, 2000). The BoNT receptor-binding module establishes the cellular specificity mediated by its high affinity interaction with a surface protein receptor and a ganglioside co-receptor (Nishiki *et al.*, 1996; Dong *et al.*, 2003; Rummel *et al.*, 2004). Given that the BoNT heavy chain forms channels in lipid bilayers (Hoch *et al.*, 1985; Donovan and Middlebrook, 1986; Blaustein *et al.*, 1987) and PC12 cells (Sheridan, 1998), predominantly under acidic conditions and only after chemical reduction, it has been surmised that the BoNT translocation module mediates the passage of the enzymatic module from the interior of the endosome into the cytosol (Hoch *et al.*, 1985; Schiavo *et al.*, 2000). Recently, we discovered that the heavy chain of BoNT acts as both a channel and a transmembrane chaperone for the light chain to ensure a translocation competent conformation during its transit from the acidic endosome into the cytosol - its site of action (Koriazova and Montal, 2003). The light chain is a Zn^{2+}-metalloprotease that cleaves the protein components involved in synaptic vesicle fusion with the neuronal membrane, thereby abrogating synaptic transmission (Schiavo *et al.*, 2000). To accomplish this task, the heavy chain operates as a transmembrane protein-conducting channel: the channel is occluded by the light chain during transit, and open after completion of translocation and release of cargo, acting intriguingly similar to the protein-conducting/translocating channels of the endoplasmic reticulum (ER), mitochondria, and chlo-

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roplasts (Koriazova and Montal, 2003). This finding has outlined a novel way of thinking about BoNT neurotoxicity, shifting the focus of attention on its translocation within cells rather than on the protease activity of the light chain, which is known not to be toxic unless it is internalized. And, it has suggested that the BoNT channel represents a potential target for intervention based on the identification of open channel blockers as a single class of drugs that would be effective against all seven *Clostridial butulinum* neurotoxin isoforms. To investigate this option we have developed a neuronal system to characterize the channel and chaperone activities of BoNT under conditions which closely emulate those prevalent at the endosome, and which are relevant to the neurotropism and neuroparalytic effects of BoNTs. Here we show that Neuro 2a neuroblastoma cells appear to be such a system.

MATERIALS AND METHODS

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Purified native BoNT isoform A was from Metabiologics (Madison, WI).

Cell Culture

Neuro 2a neuroblastoma cells, derived from a spontaneous tumor of a Strain A albino mouse, were obtained from the American Type Culture Collection and passaged in DMEM (BioWhittaker, Rockland, ME) supplemented with penicillin 10mM/streptomycin 10 μg/mL/glutamine 2mM (Invitrogen, Carlsbad, CA), and 5% newborn bovine serum (Invitrogen). Neuro 2a cells were plated onto Matrigel (BD Biosciences, San Jose, CA) coated glass coverslips at 500 cells/coverslip, and cultured at 37°C, 5% CO_2 for 1-3 days prior to patch clamp recordings.

Patch Clamp Recordings

Capillaries of borosilicate glass from Hilgenberg (Germany) were polished and used at 3.5-7.0 MΩ resistance when immersed in recording solution. Excised patches in the inside-out configuration were used (Hamill *et al.*, 1981). After gigaohm (GΩ) seal formation, the patch is excised from the cell and current recordings are obtained under voltage clamp conditions. Records were acquired and analyzed using the patch clamp amplifier system (List EPC-9, HEKA Electronik, Germany) fitted with an ITC-16 interface (Instrutech, Port Washington, NY) and the Pulse/PulseFit acquisition and analysis software (HEKA, Germany). Data were acquired at a sampling frequency > 5 kHz and filtered online to 3 kHz with a 3-pole Bessel filter. Data were analyzed using Clampfit v.9.2 software (Axon Instruments, Sunnyvale, CA), Microsoft Excel and IGOR Pro (Wavemetrics, Portland, OR). All statistical values represent means ± SEM, unless otherwise indicated. *n* and *N* denote number of experiments and number of events, respectively. All experiments were conducted at 23 ± 1°C.

Solutions

To emulate endosomal conditions the external (bath) solution contains (in mM) CsCl or NaCl 200, NaMOPS [3-(N-morpholino) propanesulfonic acid] 5, (pH 7.0 with HCl), DTT (dithiothreitol) 1, and the internal (pipet) solution contains (in mM) CsCl or NaCl 200, NaMES [2-(N-morpholino) ethanesulfonic acid] 5, (pH 5.3 with HCl), DTT 1. The osmolarity of both solutions is adjusted to ~370 mOsm. To improve seal stability in the recordings using NaCl, the reductant DTT was removed from the pipette solution and the bath solution was supplemented with 0.25mM Tris(2-carboxy-ethyl)phosphine hydrochloride (TCEP). Channel insertion is achieved by supplementing 5 μg/ml BoNT A holotoxin to the internal (pipet) solution, which is set to an endosomal pH of 5.3.

RESULTS

Neuro 2a cells constitute a good neuronal model for neurosecretion and electrophysiology. Neuro 2a cells are highly sensitive to BoNTs (Yowler *et al.*, 2002) and are readily accessible for patch clamp recordings (Hamill *et al.*, 1981). To attenuate or eliminate endogenous channel activity while selectively augmenting the detection of BoNT channel currents, the external and internal solutions contain CsCl: Cs^+ acts as the current carrier for the BoNT channel (Sheridan, 1998) and it does not permeate through endogenous K^+ or Na^+ channels (Hille, 2001). In addition, the solutions are supplemented with 1 mM $ZnCl_2$ (bath) or 1 mM 4,4-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (pipet) to eliminate currents arising from endogenous Cl^- channels known to be present in these cells (Lascola *et al.*, 1998; Carpaneto *et al.*, 1999). Under these conditions, the endogenous channel activity of Neuro 2a cells is practically abolished (n = 361 control experiments).

Toxin insertion and channel formation are pH and redox dependent; no channels are detected when the internal solution is held at pH 7 rather than pH 5.3 or when the bath solution does not contain reductant. The current flowing through individual BoNT channels at the indicated voltages is shown in figure 1. The channels are voltage dependent, opening only at negative voltages. Single-channel currents were determined

at each voltage from amplitude histograms (Keller *et al.*, 1986). The single-channel current (I)-voltage (V) curves for BoNT channels are displayed in figures 2a and 2b. BoNT channels open to a main conductance of 105 ± 5 pS and display a conspicuous subconductance of ~10 pS ($6 \le n \le 22$; N = 2,820 per data point) (FIG. 2a). Inspection of figure 1 clearly shows that the BoNT channel activity occurs in bursts interspersed between periods of little or no activity; the frequency of burst occurrence is also voltage dependent, increasing with negative voltages up to ~ -80 mV. The quiescent periods between bursts are prolonged at voltages more negative than this threshold even though the channel fast gating to the open state continues to increase up to -110mV.

Within the bursts, the channel resides preferentially in the open state (O) making frequent and fast transitions to the closed state (C). The open channel probability

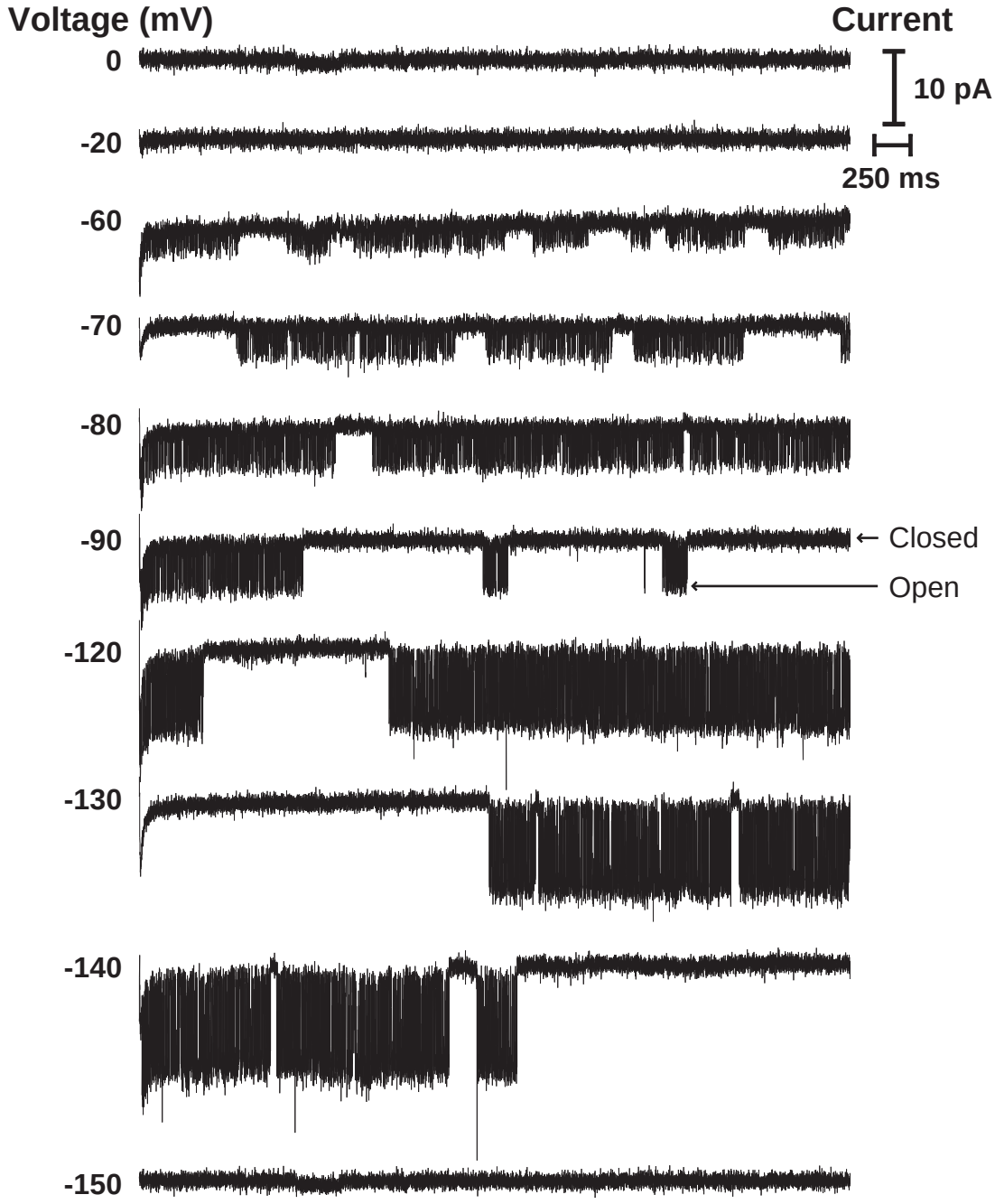

FIGURE 1 Botulinum neurotoxin A channels in excised patches of Neuro 2a cells. Representative single-channel currents at the indicated voltages; consecutive voltage pulses applied to the same patch. Channel opening is indicated by a downward deflection. BoNT A channels occur in bursts; the burst frequency and duration are voltage-dependent. Recordings were obtained in 200 mM CsCl solutions. BoNT A channels open to a main conductance of ~105 pS.

(P_o) within bursts sharply increases with negative voltages: the voltage at which $P_o = 0.5$ is 45 ± 7 mV ($6 \leq n \leq 22$; $N = 2{,}820$ per data point) (FIG. 2d).

At the onset of a burst, the channel enters the ~10 pS subconductance state (S) and then undergoes quick transitions between this intermediate state and the open state (FIG. 2b). This is clearly shown in figure 3. The open state shows very fast transitions to the substate and to the closed state giving the appearance of flickering between the open state and the substate, the predominant activity within a burst. A section delimited by the arrows is displayed at higher time resolution in the lower panel, in which transitions between the three indicated states are clearly discerned. Channel opening (O) is indicated as a downward deflection and the approximate value of the subconductance state (S) is marked. The characteristic opening and closing transitions are resolved, showing the occurrence of fast transitions to the substate. Figure 2c illustrates the transitions between a closed (C), open substate (S) and the fully open state (O). The magnitude of the arrows connecting the states is proportional to the frequency of occurrence of transitions between the three states. Within a burst, the channel rarely returns to the closed state from the open state, exhibiting a high propensity to fluctuate between the subconductance and open states or the subconductance and closed states. Thus, the subconductance state and the transitions into and out of this state are clearly recognized in the single-channel recordings. This pattern of channel activity was previously identified in single-channel recordings from tetanus toxin, another clostridial neurotoxin reconstituted in lipid bilayers (Gambale and Montal, 1988), suggesting similarities of the assembled and functional clostridial channels in membranes.

To investigate the BoNT channel properties under ionic conditions that approximate more closely those prevalent in endosomes, the BoNT channel was characterized in 200 mM NaCl and compared with the properties hitherto described in 200 mM CsCl. Representative

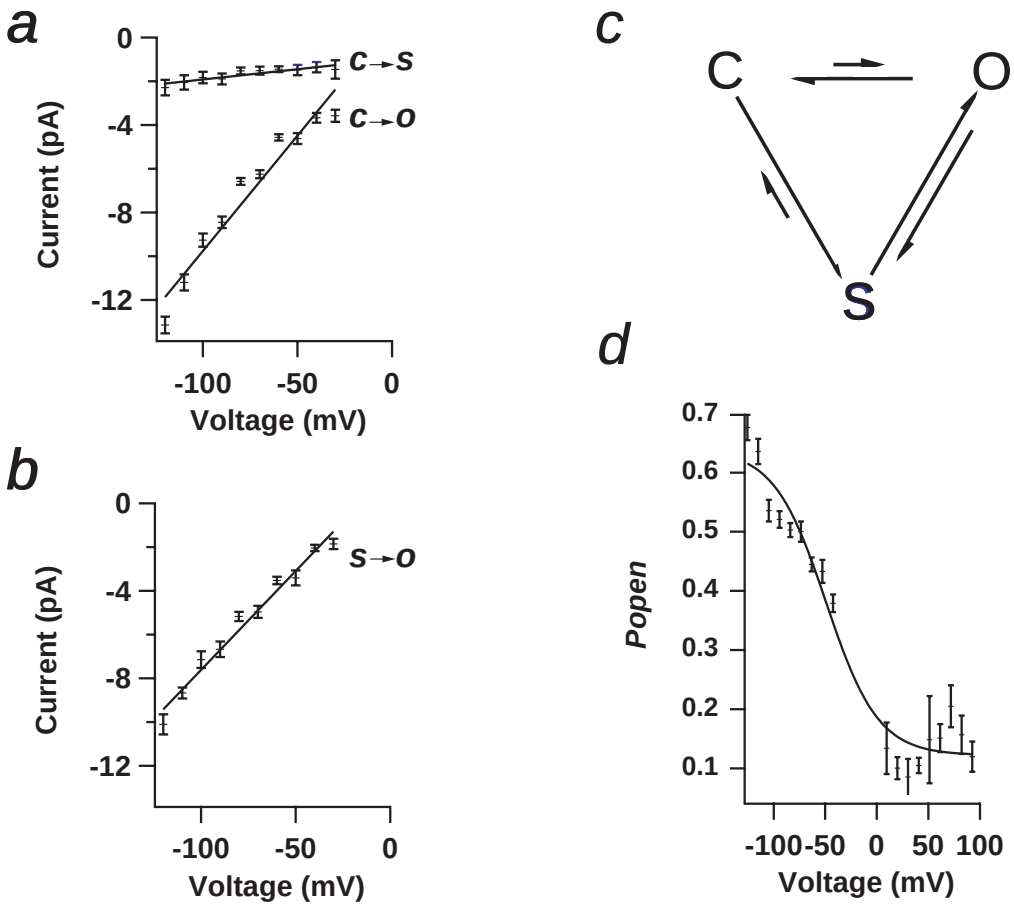

FIGURE 2 Analysis of BoNT A channels in Neuro 2a cells. (**a**) Single channel current-voltage characteristics for transitions from the closed state (C) to substate (S) with a conductance of 10 ± 2 pS and from closed state to open state with a conductance of 105 ± 5 pS (O). (**b**) Single channel current-voltage characteristics for transitions from the substate to the open state with a conductance of 95 ± 2 pS. (**c**) Transitions between closed (C), substate (S) and fully open (O) state of BoNT A channels. Arrow magnitude is proportional to probability of transition. (**d**) Probability of channel residence in the open state (P_o) as a function of voltage; the voltage at which $P_o = 0.5$ is -45 ± 7 mV. Analysis is based on single bursts of channel activity within a record; ($6 \leq n \leq 22$ per data point; the average N per data point = 2,820 events). Other conditions as in figure 1.

records obtained under these experimental conditions are shown in figure 4a. The patch was exposed to BoNT and voltage ramps from -100 mV to 100 mV with a cycle duration of 10 s were applied. In the absence of BoNT in the pipet (-) no channel activity is recorded. In contrast, when the BoNT is present inside the pipet (+), the occurrence of discrete transitions between the closed and open state of the channel are distinctly resolved. The pattern of activity is similar both in Cs^+ (middle panel) or Na^+ (lower panel). A major difference is the single channel conductance: For Cs^+, the single channel conductance is 105 ± 5 pS ($6 \leq n \leq 22$; N = 2,820 per data point) (FIG. 2) and for Na^+, 65 ± 4 pS (n = 6, N =12,000). A segment of a current record obtained at -90 mV in Na^+ is displayed in figure 4b at higher resolution. The occurrence of the subconductance state (S) of 10 ± 2 pS is clearly discerned in the single channel recording. The signal to noise ratio of these recordings is very high, therefore validating the strategy and providing a sensitive assay to characterize the properties of the BoNT channel, and modifications introduced by channel blocker candidates. The transmembrane voltage may be readily modified and a variety of pulse stimulation protocols may be applied to examine alterations ensued by these modifiers on the conduction pathway or the lifetime of the channel in open or closed states, namely the gating kinetics.

CONCLUSIONS

Remarkably, the pattern of channel activity displayed by BoNT A in Neuro 2a cells is similar to that previously characterized in lipid bilayers composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and the ganglioside GT1b (Koriazova and Montal, 2003). No other cellular components are required in order to retrieve BoNT A channel activity in lipid membranes. BoNT A holotoxin single channel conductance (γ) in planar lipid bilayers bathed in 0.2 M KCl is 74 ± 12 pS (L. Koriazova and M. Montal, unpublished results). This value is in good agreement with a γ = 65 ± 4 pS recorded in Neuro 2a cells bathed in 0.2 M NaCl, given the equivalent ionic conductance in aqueous solution for KCl = 1.18 NaCl (Hille, 2001).

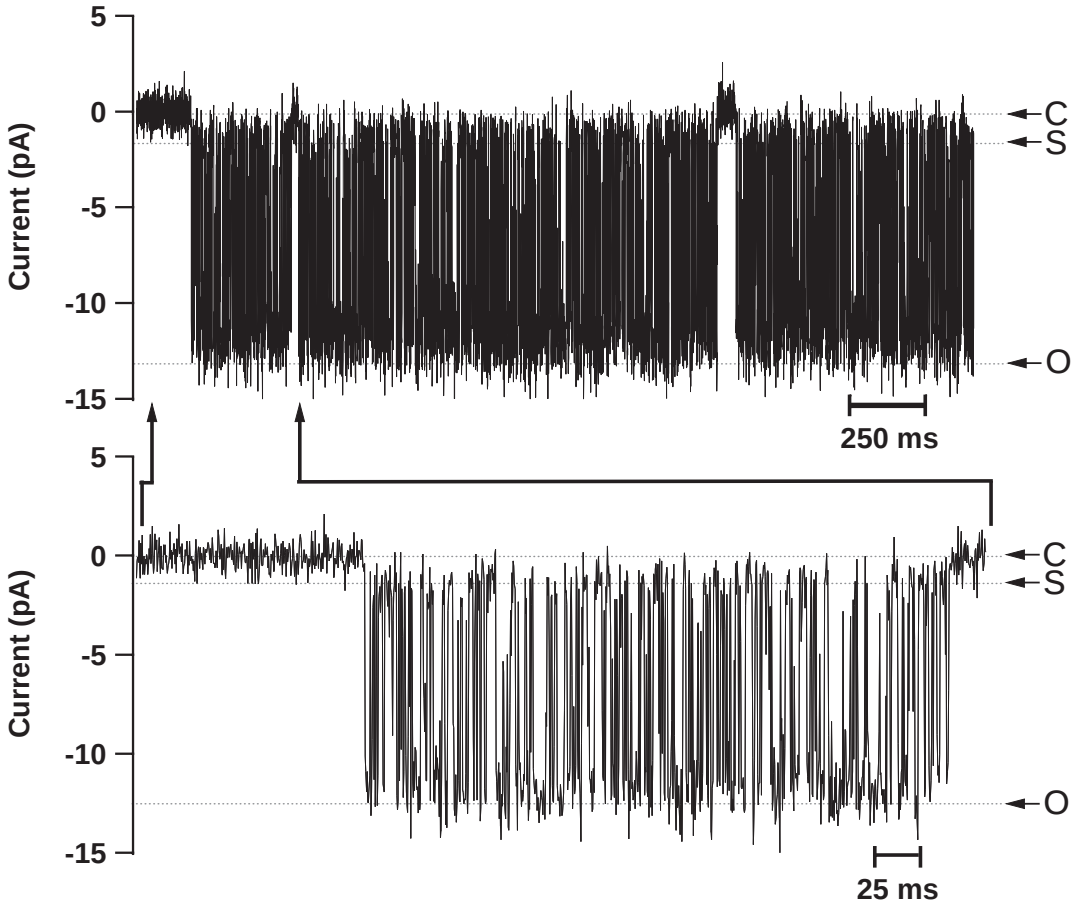

FIGURE 3 BoNT A bursting channel activity in excised patches of Neuro 2a cells. At -130 mV transitions from the closed (C) to the open (O) state and from the substate (S) to the open state are clearly resolved. The BoNT A channels open to a main conductance of ~105 pS and a subconductance of ~10 pS. The approximate current value for the subconductance state (S) is marked. The lower panel shows the section of the record delimited by the arrows at higher time resolution. Other conditions as in figure 1.

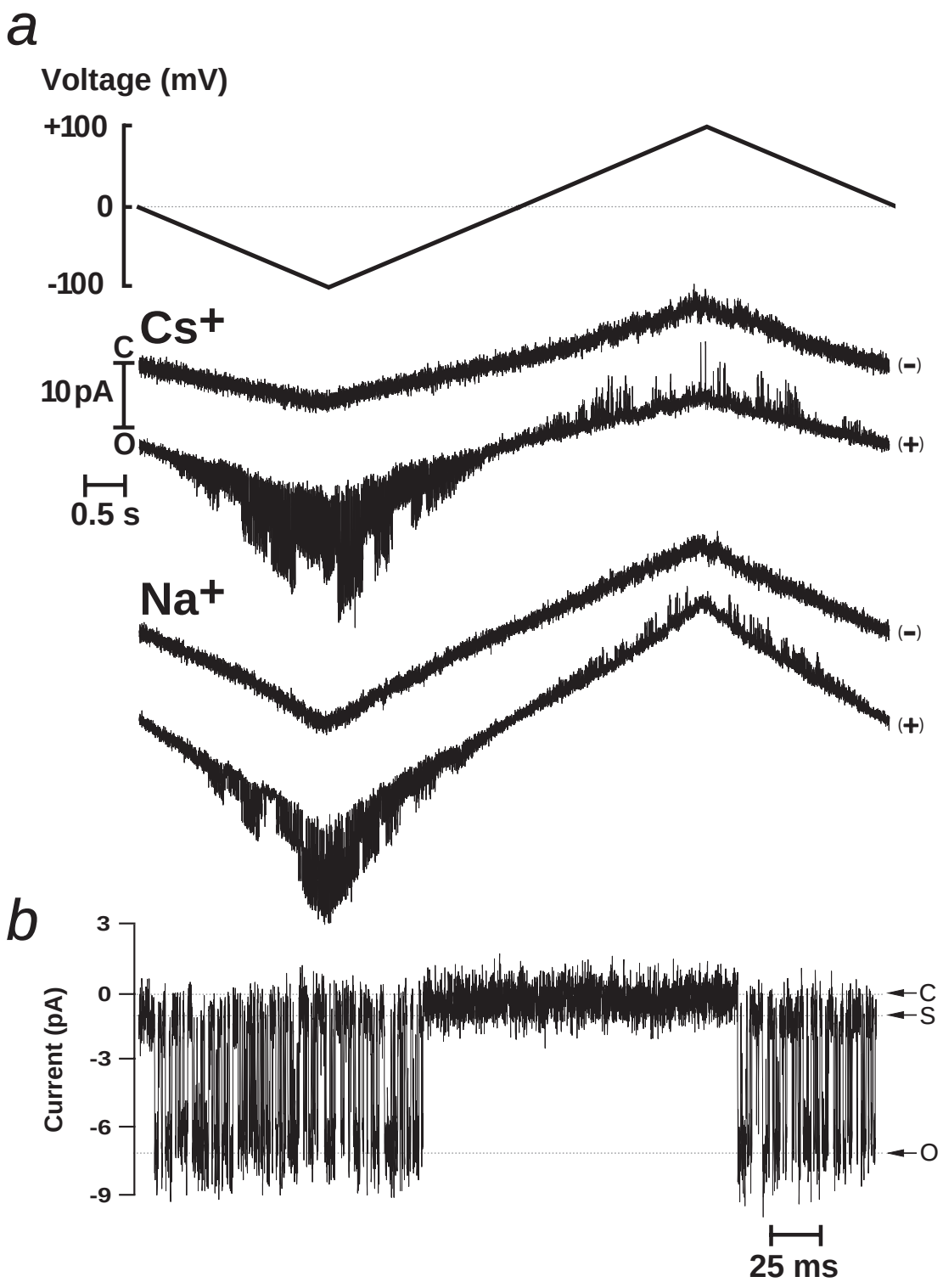

FIGURE 4a Current-voltage characteristics of BoNT A channels in Neuro 2a cells. Current records in response to a continuously cycled voltage of -100 mV to +100 mV (top panel) in symmetric 200 mM CsCl (middle panel) or NaCl (lower panel). Recordings obtained in the absence (-) or presence (+) of BoNT are indicated. Note the occurrence of two discrete channels with a single channel conductance of 105 pS in Cs^+ and 65 pS in Na^+; downward deflections indicate channel opening.

FIGURE 4b Single channel recording obtained in 200 mM NaCl at -90 mV. Transitions from the closed to the open state and from the substate to the open state are clearly resolved. The BoNT A channels open to a main conductance of ~65 pS and exhibit a subconductance of ~10 pS. Other conditons as in figure 1.

In addition, it is clear that the kinetics of the BoNT A single channel activity are modulated by the lipid composition of the bilayer; in Neuro 2a cells the mean lifetime of the channel in both closed and open states is ≤ 1 ms, whereas in lipid bilayers it is ≥ 5 ms. Membrane lipid composition is, therefore, an important regulator of channel activity as previously shown for tetanus toxin (Gambale and Montal, 1988; Rauch *et al.*, 1990).

A salient feature of the BoNT channel is that it is closed at positive voltages under conditions in which the orientation and the magnitude of the pH gradient, as well as the polarity and magnitude of the membrane potential, compare fairly well with those prevailing across endosomes: pH 5.3 and positive potential on the compartment containing the BoNT and pH 7.0 and negative potential on the opposite compartment. This suggests that the BoNT heavy chain channel would be closed in the endosome until it is gated by the BoNT light chain to initiate its translocation across the membrane into the cytosol.

The neuroblastoma cell line appears, therefore, to be a suitable system to characterize the BoNT channel and to pursue evaluation of plausible strategies for targeted drug delivery thereby minimizing the requirement for *in vivo* animal testing. In addition, the availability of sensitive assays for the light chain catalytic activity and for the heavy chain channel and chaperone activities in neuronal cells has opened new dimensions to investigate the conformational transitions of both chains linked to translocation across endosomes, the pivotal role of pH gradients, redox gradients, and electrical potentials across endosomes as driving forces underlying membrane insertion and translocation, and the requirements for light chain refolding and release at the endosome surface after translocation. These insights collectively with new findings obtained with similar modular toxins such as anthrax (Zhang *et al.*, 2004; Krantz *et al.*, 2005a,b) and diphtheria (Oh *et al.*, 1999; Ren *et al.*, 1999; Finkelstein *et al.*, 2000; Senzel *et al.*, 2000) are likely to be of fundamental significance to understanding the mechanism of protein translocation across membranes (Wickner and Schekman, 2005).

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